Suppression of angiogenesis by atmospheric pressure plasma in human aortic endothelial cells

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Atmospheric pressure plasma (APP) has been recognized as a promising tool for cancer therapy based on its ability to remove cancer cells by causing apoptosis and necrosis. However, to suppress the progression of tumors effectively, other factors also need to be controlled, including new vessel formation or angiogenesis, because angiogenesis is essential in tumor growth and progression. While the risk of metastasis is relatively low in the pre-vascularized tumor, once the tumor becomes vascularized, the potential for metastasis can increase dramatically. We show in the present study that APP can induce cell cycle arrest in endothelial cells and further suppress the angiogenesis process. These results strongly support the use of APP in cancer treatment. © 2014 AIP Publishing LLC.

Non-thermal atmospheric pressure plasma (APP) has been shown to be effective for cancer treatment by inducing cell death by necrosis or apoptosis,1–5 raising the possibility that APP can be used as a powerful tool for cancer therapy. However, to suppress the progression of tumors effectively, other factors also need to be controlled, including new vessel formation or angiogenesis, because angiogenesis is essential in tumor growth and progression.6,7 While the risk of metastasis is relatively low in the pre-vascularized tumor, once the tumor becomes vascularized, the growth and spreading of tumor are accelerated.6–8 For these reasons, suppression of angiogenesis around the tumorous tissue would make it possible to control the cancer progression more effectively. As an important extension of this, we study the effects of APP on the angiogenic ability of human aortic endothelial cells (HAECs) in the present work. The angiogenesis process is characterized by highly proliferating and motile endothelial cells, which form new sprouting vessels.9 We have therefore focused on the effects of APP on endothelial cells, under consideration of their proliferation, motility, and vessel formation ability.

We utilized single pin micro-jet plasma with 50 kHz and 970 V of applied AC power to treat HAECs, the endothelial cell model used in the present angiogenesis assay. The details of the plasma source have been reported in previous studies and also are provided in the supplementary material [Fig. S1].10,11 Cells were seeded on fibronectin coated (0.5 μg/ml) glass with 1800 cells/cm² density and the glass plate was placed inside a round petri-dish (φ = 35 mm). Prior to APP treatment, the cells were pre-incubated for 48 h to grow to 40% confluency. Since our goal is to demonstrate plasma treatment targeting blood vessels lined underneath the epithelial layer, HAECs were covered with a 3 mm thick barrier of culture medium to avoid direct contact with the plasma plume. After APP treatment for 20 min we incubated the cells for 24–72 h, allowing them to respond to the exogenous stimuli.

When we compared the control and APP treated HAECs after 24–72 h, we observed dramatic suppression of cell proliferation of APP treated cells. As shown in the phase contrast images [Fig. 1(a)], the cell density change is almost negligible in the plasma treated sample while the control increases in density over extended periods of time (24 h and 72 h). The proliferation rate was quantified by cell numbers counted from multiple phase contrast images and is plotted in Fig. 1(b). The population of the plasma treated cells is arrested during the 72 h time span, but the control cells increase about 2- and 3-fold in population, after 24 h and 72 h, respectively [Fig. 1(b)]. To check whether the observed phenomena are consequences of cell cycle arrest, we utilized flow cytometry (FACS) after propidium iodide (PI) staining to measure the portion of the cell cycles; G0/G1, S, G2/M [Fig. 2(a)]. 11,12 The collected data by FACS were analyzed by software FlowJo (Tree Star, Ashland, OR, USA) to obtain the accumulative cellular population in each cell cycle, G0/G1, S, and G2/M phase [Fig. 2(b)]. As shown in Figure 1(c), the percentage of cells in the G2/M phase population increases from 19.7% to 29.0% and 47.1%, while that of the G0/G1 phase population decreases from 72.6% to 62.4% and 40.8% in the plasma treated cells after 24 h and 72 h, respectively. On the other hand, the percentages of each phases of cell cycle are consistent over time in the control cells [Fig. 1(d)]. These results suggest the accumulation of a phase population and cell cycle arrest in the G2/M phase in plasma.
treated sample. Interestingly, this cycle arrest did not extend to a massive cellular apoptosis. For the plasma treated cells, the fraction of cells undergoing apoptosis increases from 0.5% to 2.3% over time; however, the fraction is still negligibly small, particularly compared to the control samples, where the average is about 0.9% during a 72 h time window [Fig. 1(c)].

If the cellular cycle is arrested in a certain phase, generally DNA damage is involved. This consequential relationship was confirmed by immunofluorescence images of phosphorylated histone 2AX at serine 139 (γ-H2AX) and nucleus. Figure 2(a) shows representative immunofluorescence images of control and plasma treated HAECs. The γ-H2AX, the phosphorylated form of histone 2AX (H2AX) at serine 139, is the marker of double-stranded DNA breaks. Through this marker, we can confirm whether DNA is damaged. In APP treated cells, γ-H2AX becomes visible right after the plasma treatment and persists to 72 h while it remains negative in control cells [Fig. 2(a)]. This is consistent with previous plasma studies that presented firm evidence that APP treatment can induce DNA damage. We then scanned the messenger RNA (mRNA) levels of a series of factors possibly involved in the cell cycle arrest in the G2/M phase by real-time polymerase chain reaction (PCR). In particular, the p53/p21 pathway was examined closely, because both p53 and p21 are considered potent inhibitors of cellular proliferation and are capable of arrest cells at G2/M and G1/S in a cell type dependent manner. In Figure 2(b), p53 shows a slight increase in its mRNA expression after 72 h but no significant increase at 24 h. Previous studies of p53 showed that the accumulation of activated p53 protein is a more critical factor than elevated expression of the p53 gene, and this means that p53 mRNA elevation is not necessary for the p53/p21 pathway activation. Surprisingly, as presented in Figure 2(c), our PCR data for p21, a tumor suppressor gene, show a significant increase in its mRNA expression after plasma treatment (3-fold and 10-fold increases after 24 h and 72 h, respectively). Although for regular maintenance for HAECs, the culture media should be changed every 2 days, we kept the same media for 72 h both in the plasma treated sample and the control to provide exogenous stimuli, such as the reactive oxygen species (ROS) generated from APP and conceivably dissolved into the medium to the cells. This culture condition appears to induce stress to the cells, resulting in slight increases of p53 and p21 levels in the control samples (1.5- and 2-fold, respectively) after 72 h of incubation. However, considering the negative DNA damage marker in the control cells [Fig. 2(a)], this change in mRNA level in the control HAECs is presumably a different phenomenon from that shown in plasma treated samples and can

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**FIG. 1.** Cell growth was monitored (a) in phase contrast image for control and APP treated samples at 0 h, 24 h, and 72 h after plasma treatment [Scale bar = 200 μm] and (b) number of cellular population of control and plasma treated HAECs at 0 h, 24 h, and 72 h. Progression through the cell cycle was measured by FACS with the cells collected from the control and APP treated samples at 0 h, 24 h, and 72 h. (c) Cellular DNA content histograms showing the cell cycle phase distribution of HAEC. The positions of G0/G1, S, and G2/M are marked. (d) The fractions of cells (%) at each phase of the cell cycle are listed.
be ignored. Overall, through the positive result for the DNA damage marker and a significant increase of p21 in APP treated cells, we can speculate that plasma-induced suppression of cell proliferation was mediated by a signaling cascade through the p53/p21 pathway.

From Figure 3(a), we also can see a morphological change in plasma treated cells, which was confirmed by labeling cells for actin stress fiber. The cells in the plasma treated sample grow larger and become rounded in shape while the control cells remain unchanged over time [Fig. 3(a)]. Actin stress fibers in the periphery of the cell are intact or even become stronger over time but in the middle of the cytosol the actin stress fibers become thinner and diminished after 24 h and almost disappeared after 72 h [Fig. 3(a)]. These morphological changes are schematically illustrated in Figure 3(b) as insets in the graph, and indicate a less motile phenotype of HAECs.19,20

To further investigate whether these phenotypic changes in HAECs are linked with the cellular motility, we monitored the cell trajectory to conduct a quantitative evaluation of cell migration. Based on the phase contrast images taken every 5 min, cells were tracked down by the cell tracking program in ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). Accumulative path length of every 5 min was obtained from the trajectory of control and plasma treated cells [Fig. 3(b)]. The effects of APP on the mobility of HAECs were examined at two different time points, first at 0 h and then at 24 h after plasma treatment. Even with the growing cellular population and less empty space to move after 24 h, the control cells show almost no change in the total path length [Fig. 3(c)] at 0 h and 24 h. In contrast, the total path length of the plasma treated cells significantly decreased by 50% in comparison to the baseline [Fig. 3(c)], which could be predicted from the previous morphological changes to a less motile phenotype [Fig. 3(b)].

In order to examine whether this observed reduction in both proliferation and motility further suppresses the angiogenic ability of endothelial cells by APP treatment, we performed an in vitro tube assay with both the plasma treated HAECs and control HAECs. Cells were collected from the glass surface to be re-plated on Matrigel (BD Biosciences, cat#356234), whereupon the endothelial cells can form a vascular tube network,21 after 24 h of plasma treatment. The tube formation process was monitored for about 9 h by image microscopy. Phase contrast images of both the plasma treated HAECs and the control HAECs were taken after re-plating and revealed that APP treated cells formed vessels with less integrity. We characterized the integrity of the tube network by the number of complete loops and the number of open-ended branches. Base on this observation, it can be concluded that the tube network is more robust when it has more complete loops and fewer open-ended branches. The complete loops are depicted as polygons and the white arrows indicate open-ended branches in Figure 4(a). In the Matrigel where APP treated HAECs were seeded, the tubes are thinner and looser compared to the control sample [Fig. 4(a)]. When we quantified the integrity, plasma treated HAECs showed 3-fold fewer complete loops [Fig. 4(b)] and 3-fold more open-ended branches [Fig. 4(c)], indicating significantly delayed vessel formation, or suppressed angiogenesis.

Our study shows that APP suppresses the vessel forming capability of endothelial cells by arresting cell cycle and reducing their motility. Reduction in both cellular proliferation and motility is very critical in anti-angiogenesis but this is not only the path through which the angiogenesis can be suppressed. We also elucidate that this inhibition of angiogenesis is caused by APP induced DNA damage. Moreover, the DNA damage and consequential cell cycle arrest were

FIG. 2. (a) Immuno-fluorescence images of phosphorylated histone 2AX at serine 139 (γ-H2AX) (green, white arrows) and nucleus (blue) of control and plasma treated cells at 0 h, 24 h, and 72 h [Scale bar = 50 μm]. Gene expression level of (b) p21 and (c) p53 in HAECs measured by real-time PCR (*p < 0.01, **p < 0.01).
found to be mediated by p53/p21 pathway, distinctly from currently used anti-angiogenic drugs, most of which work by modulating either vascular endothelial growth factor receptor (VEGFR) or epidermal growth factor receptor (EGFR) and their downstream pathways. While it is difficult to compare APP with other anti-angiogenic drugs directly, APP is comparable to surgical tools rather than drug treatments because APP only affects the treated area, which, we believe, can be a huge advantage over the currently used drugs. Anti-angiogenic drugs are designed to suppress formation of new vessels around tumors but they can also affect endothelial cell types across the entire body. In fact, the commercialized drugs for anti-angiogenesis therapy have been reported to have side effects such as increased internal bleeding, holes in intestines, increased blood pressure, retarded wound healing, and severe defects in fetus development. These side effects can sometimes be fetal. In case of APP, we can select the target area and treat the tissue without affecting the rest of the body.

Overall, although it is difficult to compare the efficacy of APP with commercialized drugs side by side, because the approach of the treatment and the acting mechanism seem to be different from each other, we are very optimistic about
the potential usage of APP in cancer treatment. Tumor is very complex. In addition to the cancerous cell mass, the microenvironment of the tumor contains many different cell types including macrophages, fibroblasts, and endothelial cells. In particular, a large number of endothelial cells are recruited to the tumor site to supply nutrients by forming new vessels through the process called angiogenesis. Therefore, APP can not only induce the deaths of cancer cells at the tumor but also suppress the angiogenesis, producing synergistic effects.

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11See supplementary material at http://dx.doi.org/10.1063/1.4870623 for text detailing of experimental setup, methods of cell culture and additional supporting data.